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13. Abstract *(Maximum 200 Words)* (abstract should contain no proprietary or confidential information)

The Brn-3b POU family transcription factor is over-expressed in breast cancer cells compared to normal mammary cells. In this project, we have stably transfected the human breast cancer cell line MCF-7 with vectors expressing the sense or anti-sense strand of Brn-3b so as to isolate cell lines with respectively enhanced or reduced Brn-3b levels. The cell lines with enhanced Brn-3b levels show enhanced growth rate, saturation density, cell division, anchorage independence *in vitro* and enhanced ability to form tumors *in vivo*. In contrast, the cell lines with reduced Brn-3b show reductions in all those growth parameters. In addition, the cell lines with altered Brn-3b levels show altered expression of genes such as BRCA-1 and the estrogen receptor whose expression is known to be modulated in breast cancer. These findings thus establish Brn-3b as a key factor regulating gene expression and growth of human breast cancer cells.

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Table of Contents

Regulation of BRCA-1 Gene Expression and Mammary Tumorigenesis by the Brn-3b POU Family Transcription Factor

Introduction

This project is based on our finding that the Brn-3b POU family transcription factor is over-expressed in breast cancer cells compared to its expression in normal mammary epithelial cells. Moreover, prior to the start of the project, we demonstrated that over-expression of Brn-3b correlated with reduced expression of the BRCA-1 anti-oncogene in the breast tumour cells (1). The aim of this project was therefore to evaluate the role of Brn-3b in regulating the general growth and gene expression pattern of breast cancer cells and its role in particular in regulating BRCA-1 gene expression.

In our first year report, we reported progress in the areas corresponding to the two major tasks of the project. Task 1 related to an analysis of the mechanisms by which Brn-3b inhibits the BRCA-1 promoter. With respect to this task, we reported the mapping of the region of the BRCA-1 promoter which is inhibited by Brn-3b and also by the short form of the related factor Brn-3a. Moreover, we demonstrated for the first time that the long form of Brn-3a can activate the BRCA-1promoter.

The second task of the project concerns the effect of manipulating Brn-3b expression on the expression of BRCA-1 and the proliferation of normal and malignant mammary cells. With respect to this task we reported the construction of cells lines derived from the human breast cancer cell line MCF-7 which respectively either over-express or under-express Brn-3b following transfection with expression vectors expressing either the sense or anti-sense strand of Brn-3b. We reported the exciting results that the cells over-expressing Brn-3b showed much more rapid growth and an enhanced saturation density compared to the control cells. In

contrast, the cells with reduced expression of Brn-3b showed decreased growth rate and saturation density compared to the control cells. Hence, these findings directly demonstrate the key role of Brn-3b in regulating the growth of breast cancer cells.

Body

In view of the importance of our cell lines in demonstrating the effect of Brn-3b on the growth of breast cancer cells, we have devoted our efforts in the past year to the further characterisation of these cells as outlined in Task 2, Sub-Tasks 4-6 of the original proposal. This emphasis on Task 2 was specifically noted by the referee of our last report and was approved of. In addition, we have also begun to use global analysis of gene expression in the manipulated MCF-7 cells to further characterise the effect of Brn-3b on a range of target genes. Correspondence with Grants Administration Officer indicated that this could be regarded as an extension of Task 2, Sub-Task 4 not requiring any change to the POW.

Task 2, Sub-Task 4

As envisaged in the original application, we have compared the expression of several genes in the MCF-7 cell lines respectively over-expressing or under-expressing Brn-3b. These genes were chosen for potential importance in human breast cancer. Expression was analysed both at the mRNA level by reverse transcriptase/polymerase chain reaction (RT-PCR) and at the protein level by western blotting. As expected, enhanced expression of Brn-3b was observed in the anti-sense cell lines expressing reduced levels of Brn-3b whilst over-expression of BRCA-1 was observed in the cells over-expressing Brn-3b. Conversely, Brn-3b over-expressing cells showed enhanced expression of both the estrogen receptor, human chorionic gonadotrophin and of the 27kDA heat shock protein (HSP27) all of which have been reported to be over-expressed in breast cancer. As a control, we observed no change in

the expression of other heat shock proteins HSP70 and HSP90. Typical results of this procedure are illustrated in Fig. 1.

These findings therefore establish that alteration of Brn-3b as well as altering the growth rate of breast cancer cells (see below) also alters the expression of specific genes which are known to be altered in breast cancer. To follow this up further, we used a global analysis of gene expression in which cDNA prepared from mRNA of the Brn-3b over-expressing or under-expressing cells was used to probe the Atlas human cancer cDNA array which contains sequences from 1176 cancer-related genes arrayed on a filter. The results of this experiment (Table I) showed clear alteration in the expression of 51 genes whose expression was either up- or down-regulated in the Brn-3b over-expressing cells versus the under-expressing cells. These genes include a number of different genes whose expression is important in cellular growth and differentiation and hence their identification as potential targets for Brn-3b is of great interest. This aspect will therefore be investigated further. Firstly, we will confirm the alteration in expression of these genes in our various cell lines both by RT-PCR and also by western blotting analysis using appropriate antibodies. Subsequently, we will determine whether the expression of these genes in breast cancer tissue is altered in a manner which parallels the alteration of Brn-3b expression thereby establishing them as likely candidates for regulation by Brn-3b in mammary tumour cells.

Task 2, Sub-Task 5

In this aspect of the work, we aimed to characterise the *in vitro* growth properties of the cells over-expressing or under-expressing Brn-3b. Partial completion of this Sub-Task was reported in the previous report where we demonstrated enhanced growth and saturation density of the Brn-3b over-expressing cells and reduced growth and saturation density of the cells with reduced Brn-3b.

As these experiments were carried out by counting cells, in the current year we followed this up by measuring the rate of cell division in the cells using the incorporation of tritiated thymidine into the cells. As illustrated in Fig. 2, the Brn-3b over-expressing cells (cell lines Z and Y) exhibited enhanced cell division compared to the control cells (cell lines B and C). Moreover, the anti-sense cell lines with reduced expression of Brn-3b (cell lines A1 and A2) showed correspondingly reduced cell division. Hence, these results confirm and extend our earlier observations on the role of Brn-3b in controlling the proliferation of breast cancer cells.

To extend these studies, we also determined the ability of the various cells to form colonies in soft agar. This is a measure of their ability to grow in an anchorage-independent manner which is predictive of tumour growth *in vivo*. In these experiments (Fig. 3) the Brn-3b over-expressing cell line (Z) showed statistically significantly enhanced ability to form colonies compared to the control cells. Most importantly, the anti-sense cell clone (A1) showed a very dramatically reduced ability to form colonies in soft agar. Hence, anchorage independence is dramatically reduced in the absence of high level expression of Brn-3b.

These studies therefore indicate the key role of Brn-3b in the ability of human breast cells to grow in an anchorage-independent manner *in vitro*. As this is believed to be associated with the ability to form tumours *in vivo*, we therefore investigated this aspect as described in the next section. Further characterisation of the properties of our cells *in vitro* is continuing. In particular, as suggested by the referee on the first year report, the ability of Brn-3b to interact with the estrogen receptor and after its activity (2) suggest that altering Brn-3b may affect the estrogen responsiveness of these cells as well as altering the expression of the estrogen receptor (see above). Hence, we are now investigating whether the estrogen responsiveness of the MCF-7 cells is altered by enhanced or reduced Brn-3b levels.

Task 2, Sub-Task 6

Following on from our studies on the anchorage-independence of our cell lines *in vitro*, we have begun to investigate whether corresponding alterations in tumour growth *in vivo* can be observed.

In initial experiments, the Brn-3b over-expressing cells were inoculated into nude mice and their ability to form tumours compared to that observed with cells in which Brn-3b expression had not been manipulated. In initial experiments (Table II) clearly enhanced growth was observed for tumours derived from the Brn-3b over-expressing cells compared to tumours formed by the control cells. These experiments establish for the first time the fact that over-expression of Brn-3b can enhance tumour cell growth *in vivo* as well as *in vitro*. We are now extending these experiments using further animals and directly comparing the growth of Brn-3b over-expressing cells with that of the Brn-3b under-expressing cells as well as with control cells.

Key Research Accomplishments

- Demonstrated that the expression of specific genes such as BRCA-1, estrogen receptor, chorionic gonadotrophin and HSP27 is altered in the cells with altered Brn-3b expression.

- Used global analysis of gene expression to identify further genes whose expression is increased or decreased in response to altered Brn-3b expression.

- Demonstrated that MCF-7 cells with altered Brn-3b levels show corresponding alterations in the rate of cell division and the ability to grow in an anchorage-independent manner *in vitro* extending our earlier work on their growth rate and saturation density.

- Demonstrated that Brn-3b over-expressing cells grow more rapidly as tumours *in vivo* in nude mice compared to control cells.

Reportable Outcomes

- A paper reporting the effect of Brn-3b on the growth rate of MCF-7 cells has been submitted for publication.

- MCF-7 cell lines over-expressing Brn-3b or exhibiting reduced Brn-3b levels have now been extensively characterised.

- A number of genes whose expression is affected by altered Brn-3b levels in the MCF-7 cells have now been identified both by testing individual genes known to show altered expression in breast cancer and by a global gene expression analysis.

Conclusions

Our work in the first two years of the project has conclusively demonstrated that Brn-3b plays a critical role in regulating the growth of breast cancer cells and in determining their pattern of gene expression. Over-expression of Brn-3b enhances the growth of a human breast cancer cell line both *in vitro* and *in vivo* and alters the expression of a number of genes known to exhibit altered expression in human breast cancer. Conversely, reduced expression of Brn-3b slows the growth of cells and strongly reduces their ability to grow in an anchorage-independent manner as well as also altering the expression of a number of different genes.

The findings suggest that Brn-3b is a critical mediator controlling the growth of breast cancer cells by modulating the expression of other target genes. Future experiments will focus particularly on the effects of manipulating this factor on tumour growth *in vivo* and on further characterising the altered patterns of gene expression which it produces in the cell lines and relating these to the corresponding expression pattern of Brn-3b itself and of its target genes in patient breast cancer samples. These results are providing important information on the role of Brn-3b in regulating gene expression and cellular growth in breast cancer cells and also

establish it as a potential target for therapeutic interventions aimed at reducing its expression and/or activity in order to reduce the growth of breast tumours.

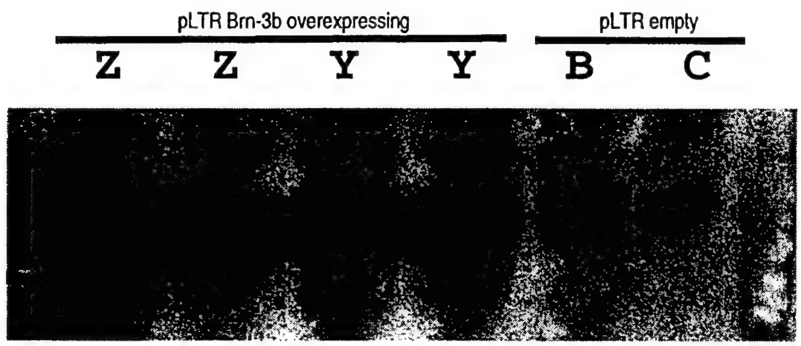
References

1. Budhram-Mahadeo, V. S., Ndisang, D., Ward, T., Weber, B. L., and Latchman, D. S. The Brn-3b POU family transcription factor represses expression of the BRCA-1 anti-oncogene in breast cancer cells. Oncogene 18: 6684-6691, 1999.

2. Budhram-Mahadeo, V. S., Parker, M., and Latchman, D. S. The POU domain factors Brn-3a and Brn-3b interact with the estrogen receptor and differentially regulate transcriptional activity via an ERE. Molecular and Cellular Biology 18: 1029-1041, 1998.

Figure 1. Western blot analysis of beta human chorionic gonadotropin in clonal MCF7 cell lines with altered levels of Brn-3b.

Immunoblots to detect the levels of beta-hCG were carried out using six clones: two overexpressing pLTR Brn-3b (Z and Y) each in duplicate, and two empty pLTR vector controls (B and C). Total cellular protein (60 micrograms per lane) was fractionated on a SDS/15% polyacrylamide gel, transferred to nitrocellulose, and probed with an anti-beta-hCG antibody. Expression levels were normalized to total protein densitometry of the commassie satined gel after western blotting. The level of beta-hCG antigen in extract from the pLTR overexpressing clones was 5.66 - 14.45 times the average endogenous level seen in the vector controls.



CLONE	DENSITOMETRY(arbitrary units)		FOLD EXPRESSION	
	beta hCG	Commassie	raw	normalized
pLTR Brn-3b short Z	1114033	551569	11.55	3.58
pLTR Brn-3b short Z	1047134	453077	10.86	4.10
pLTR Brn-3b short Y	1393001	350176	14.45	7.06
pLTR Brn-3b short Y	1255212	393675	13.02	5.66
pLTR B	130236	217177	1.35	1.06
pLTR C	62588	118621	0.65	0.94

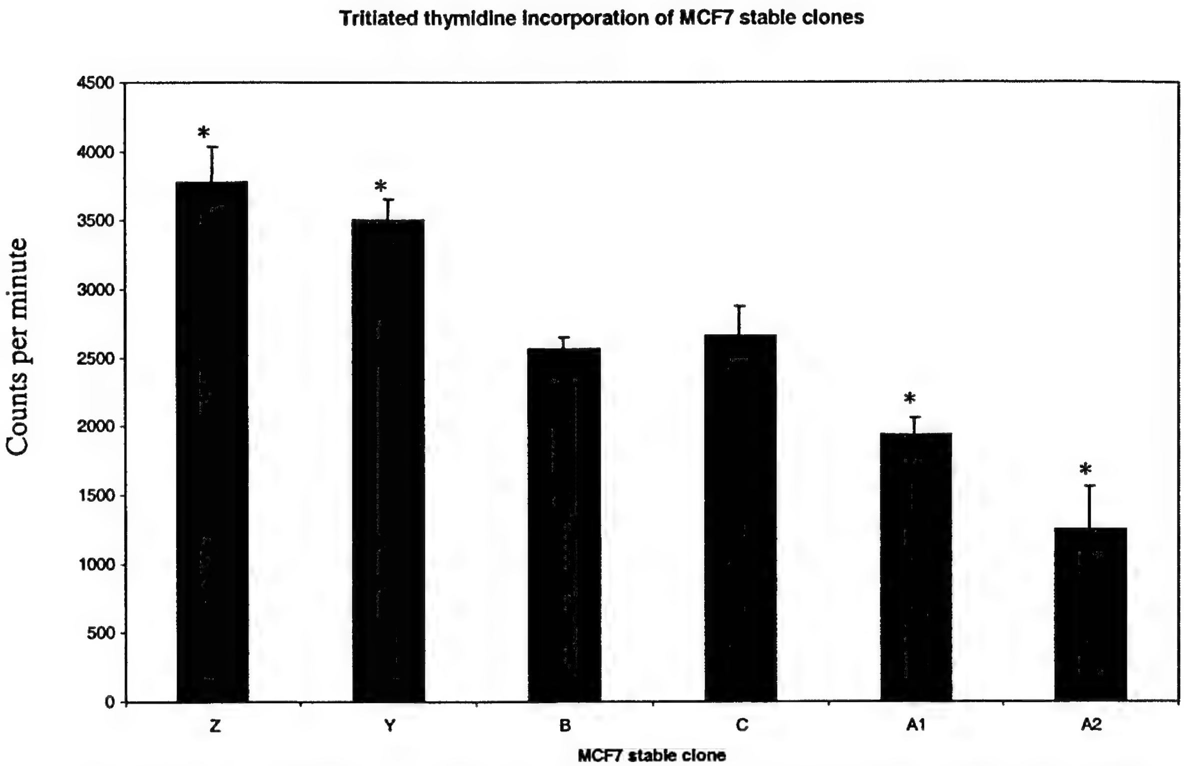


Figure 2. Tritiated thymidine incorporation by clonal MCF7 cell lines with altered levels of Brn-3b.

Cells were grown in full growth medium for 48 hours and subsequently treated with tritiated thymidine. After one hour stimulation, cells were trypsinized, harvested onto glass filters, and counts per minute from the glass filters were recorded by a scintillation counter. The counts per minute from each cell line represent the mean of three independent experiments counted in triplicate +/- the standard deviation of the mean. "*" denotes p-value less than 0.05 resulting from a chi-squared test comparing all values at the indicated time point from the experimental (Brn-3b short or Brn-3b antisense) with the expected (pLTR control).

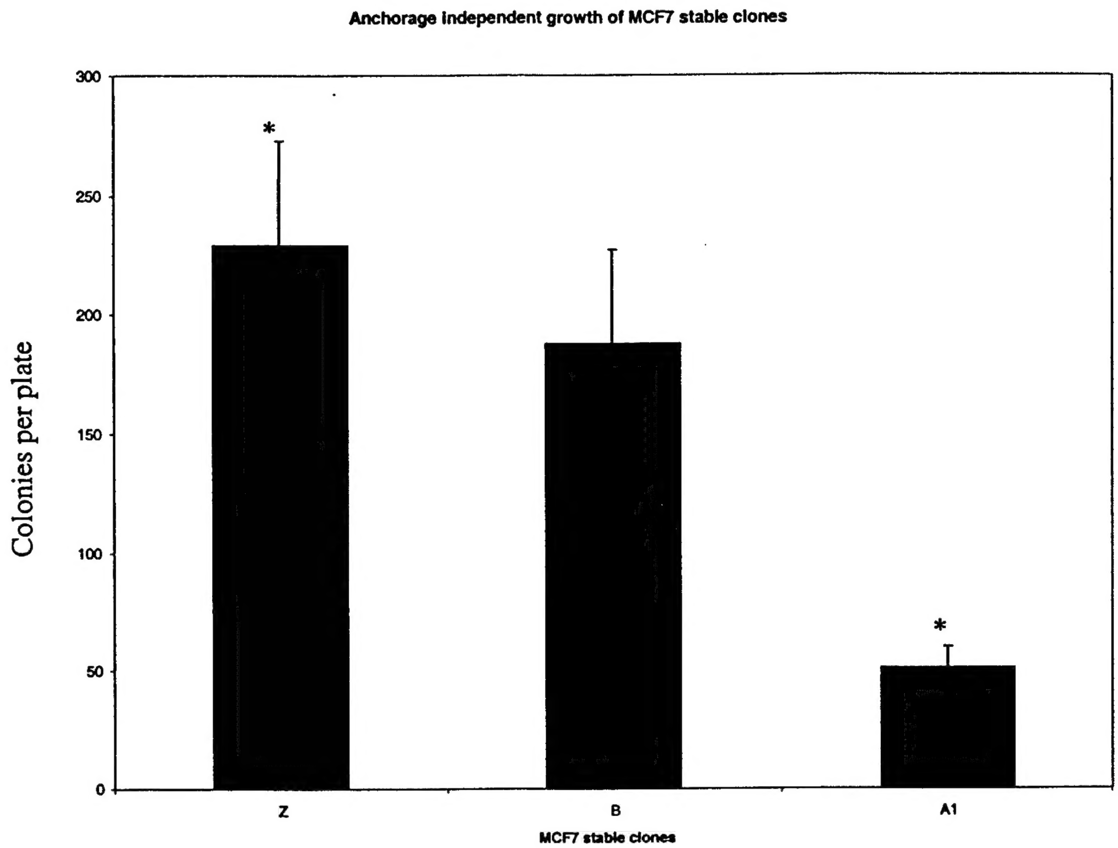


Figure 3. Anchorage independent colony formation by clonal MCF7 cell lines with altered levels of Brn-3b.

Cells were plated in full growth 0.3% agarose medium. After 21 days, colonies comprised of at least 32 cells were counted. The number of colonies from each cell line represent the mean of three independent experiments counted in triplicate +/- the standard deviation of the mean. "*" denotes p-value less than 0.05 resulting from a chi-squared test comparing all values at the indicated time point from the experimental (Brn-3b short or Brn-3b antisense) with the expected (pLTR control).

Table I. Results of the differential display of genes on the Atlas human cancer cDNA array.

http://atlas.cl

experimental		control	
brn-3b short	vs.	anti-sense	
Normalization		Thresholds	
method	coefficient	ratio	difference
global (sum)	1.30	1.69	15

Array lot#
9100044
Atlas Array
Cancer 1.2k Array

#	coordinate	Spot Intensity anti-sense	Spot Intensity brn-3b short	Ratio	Difference	RATIO UP	RATIO DOWN	? = weak signal (low trust)	Gene
1	A01c	66	28	0.42	-38		2.4		c-jun proto-oncogene; transcription factor AP-1
2	A01h	3	25	8.33	22	8.3			interferon-inducible protein 9-27
3	A03c	71	36	0.51	-35		2.0		c-myc oncogene
4	A03g	256	533	2.08	277	2.1			c-myc binding protein MM-1
5	A04j	51	88	1.73	37	1.7			cell division protein kinase 4; cyclin-dependent kinase 4 (CDK4); PSK-J3
6	A09l	50	116	2.32	66	2.3			cyclin-dependent kinase inhibitor 1 (CDKN1A); melanoma differentiation-associated protein 6 (MDA6); CDK-interacting protein 1 (CIP1); WAF1
7	A10k	84	142	1.69	58	1.7			cyclin-dependent kinase regulatory subunit 1 (CKS1)
8	A12j	44	24	0.55	-20		1.8		cdc2-related protein kinase PISSLRE
9	A12n	35	10	0.29	-25		3.5		G1 to S phase transition protein 1 homolog; GTP-binding protein GST1-HS
10	B02a	381	220	0.58	-161		1.7		ADP/ATP carrier protein
11	B02i	49	23	0.47	-26		2.1		protein phosphatase 2C gamma
12	B04j	68	129	1.90	61	1.9			rhoC (H9); small GTPase (rhoC)
13	B04n	170	288	1.69	118	1.7			B-cell receptor-associated protein (hBAP)
14	B05l	35	18	0.51	-17		1.9	?	calmodulin 1; delta phosphorylase kinase
15	B07m	41	3	0.07	-38		13.7		zyxin + zyxin-2
16	B10c	30	51	1.70	21	1.7			c-jun N-terminal kinase 2 (JNK2); JNK55
17	B10m	167	37	0.22	-130		4.5		junction plakoglobin (JUP); desmoplakin III (DP3)
18	B13j	29	10	0.34	-19		2.9	?	guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-10 subunit
19	C01g	46	19	0.41	-27		2.4		DNA ligase I; polydeoxyribonucleotide synthase (ATP) (DNL1) (LIG1)
20	C04b	127	237	1.87	110	1.9			tumor necrosis factor type 1 receptor associated protein (TRAP1)
21	C04g	34	19	0.56	-15		1.8	?	DNA excision repair protein ERCC1
22	C06n	27	10	0.37	-17		2.7	?	interferon regulatory factor 3 (IRF3)
23	C08l	27	7	0.26	-20		3.9	?	retinoic acid receptor alpha 1 (RAR-alpha 1; RARA) + PML-RAR protein
24	C12i	40	5	0.13	-35		8.0		TIS11B protein; EGF response factor 1 (ERF1)
25	C12j	28	5	0.18	-23		5.6		early growth response protein 1 (hEGR1); transcription factor ETR103; KROX24; zinc finger protein 225; AT225
26	C14m	25	3	0.12	-22		8.3		fuse-binding protein 2 (FBP2)
27	C14n	49	24	0.49	-25		2.0		transcription factor erf-1; AP2 gamma transcription factor
28	D06e	60	32	0.53	-28		1.9		integrin beta 4 (ITGB4); CD104 antigen
29	D07b	65	31	0.48	-34		2.1		high mobility group protein HMG2
30	D08f	37	11	0.30	-26		3.4		paxillin
31	D09d	44	19	0.43	-25		2.3		alpha1 catenin (CTNNA1); cadherin-associated protein; alpha E-catenin
32	D09m	55	109	1.98	54	2.0			glutathione-S-transferase (GST) homolog
33	D14i	19	3	0.16	-16		6.3	?	cysteine-rich fibroblast growth factor receptor ; Golgi membrane sialoglycoprotein MG160 (GLG1)
34	E02n	55	10	0.18	-45		5.5		78-kDa glucose regulated protein precursor (GRP 78); immunoglobulin heavy chain binding protein (BIP)
35	E03j	780	384	0.49	-396		2.0		cathepsin D precursor (CTSD)
36	E07f	130	64	0.49	-66		2.0		interleukin-1 beta precursor (IL-1 ; IL1B); catabolin
37	E07h	239	440	1.84	201	1.8			macrophage migration inhibitory factor (MIF); glycosylation-inhibiting factor (GIF)
38	E09h	25	9	0.36	-16		2.8	?	jagged2 (JAG2)
39	F04k	61	29	0.48	-32		2.1		60S ribosomal protein L5
40	F05e	14	36	2.57	22	2.6			ornithine decarboxylase
41	F08j	30	15	0.50	-15		2.0	?	HSC70-interacting protein; progesterone receptor-associated P48 protein
42	F08k	28	9	0.32	-19		3.1	?	eukaryotic translation initiation factor 3 beta subunit (EIF-3 beta); EIF3 P116
43	F08m	75	19	0.25	-56		3.9		PM5 protein
44	F10b	24	9	0.38	-15		2.7	?	IMP dehydrogenase 1
45	F10j	110	64	0.58	-46		1.7		suppressor for yeast mutant
46	F12d	25	10	0.40	-15		2.5	?	uridine 5'-monophosphate synthase (UMP synthase)
47	F12f	141	77	0.55	-64		1.8		type II cytoskeletal 2 epidermal keratin (KRT2E); cytokeratin 2E (K2E; CK2E)
48	F13k	52	15	0.29	-37		3.5		glycyl tRNA synthetase
49	F14b	24	42	1.75	18	1.8			aminoacylase 1 (ACY1)
50	G27	2180	5230	2.40	3050	2.4			liver glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
51	G43	1004	397	0.40	-607		2.5		cytoplasmic beta-actin (ACTB)

N/C = not calculated due to manually-determined inconsistencies (signal bleeding, background, etc.) in one or both spots

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TABLE II

Growth of a MCF-7 clonal cell line over-expressing Brn-3b (Z) in nude mice compared to that of a control MCF-7 cell line with normal Brn-3b levels (cell line F)

MEASUREMENTS								
Date	**24.4**	**30.4**	**8.5**	**15.5**	**21.5**	**29.5**	**4.6**	**11.6**
Tumour Z	0.49	0.64	0.88	0.88	1.00	1.15	1.30	
Tumour F	0.03	0.09	0.06	0.06	0.10	0.08	0.10	0.15